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(58) Field of Search
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(54) **Assay to detect peroxisome proliferator activity**

(57) A cell based transactivation assay, useful in detecting peroxisome proliferator activity of chemical entities, comprises co-transfecting a host cell with a full-length peroxisome proliferator activated receptor cDNA sequence and a combination of a peroxisome proliferator-responsive element, such as that of the rat acyl-CoA oxidase gene promotor, upstream from a promotor linked to a reporter gene, such as chloramphenicol acetyl transferase, CAT. This assay allows detection of peroxisome proliferator activity while employing a minimal quantity of test compound. Cells comprising such constraints are also claimed.

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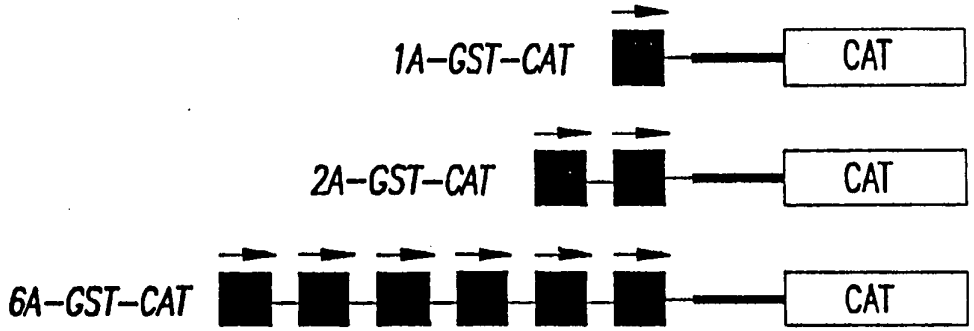
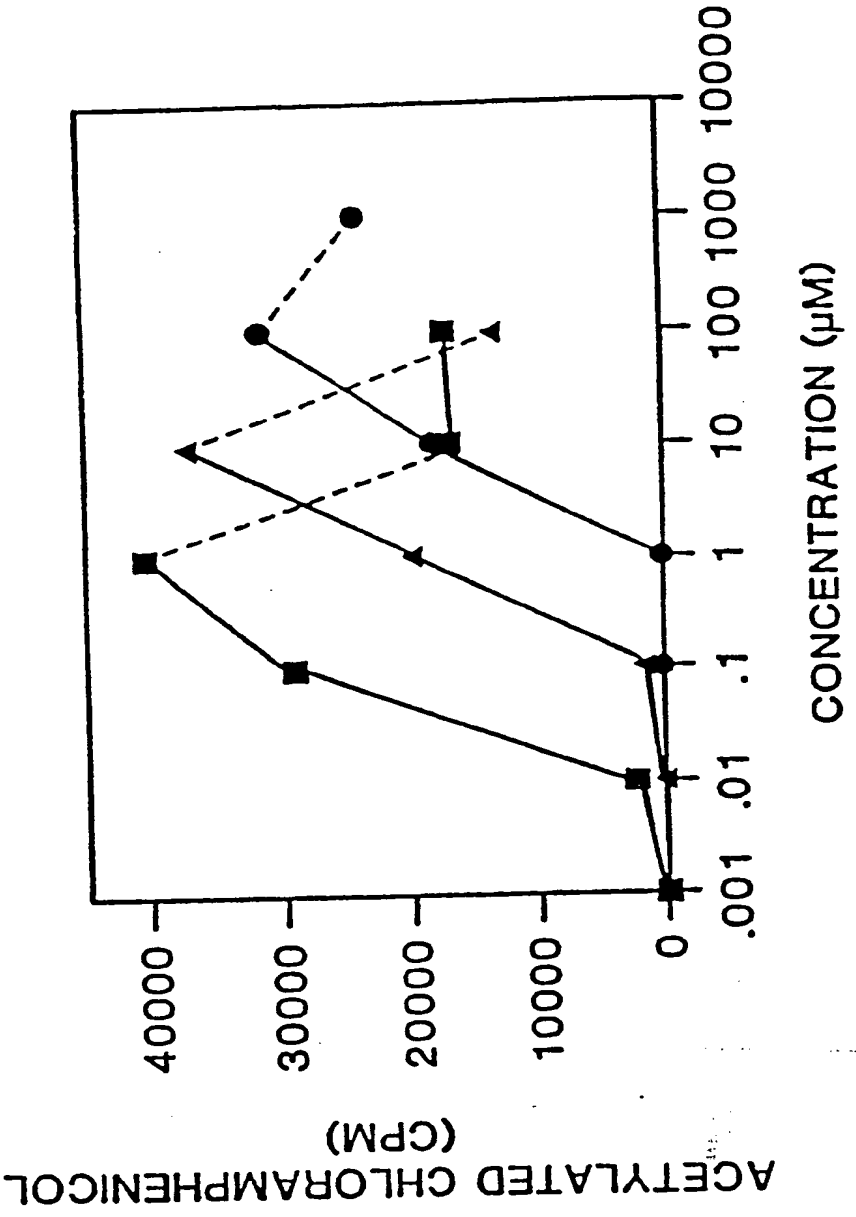


FIG.1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.1
1
10
100
1000
10000
CONCENTRATION (μM)

FIG.2

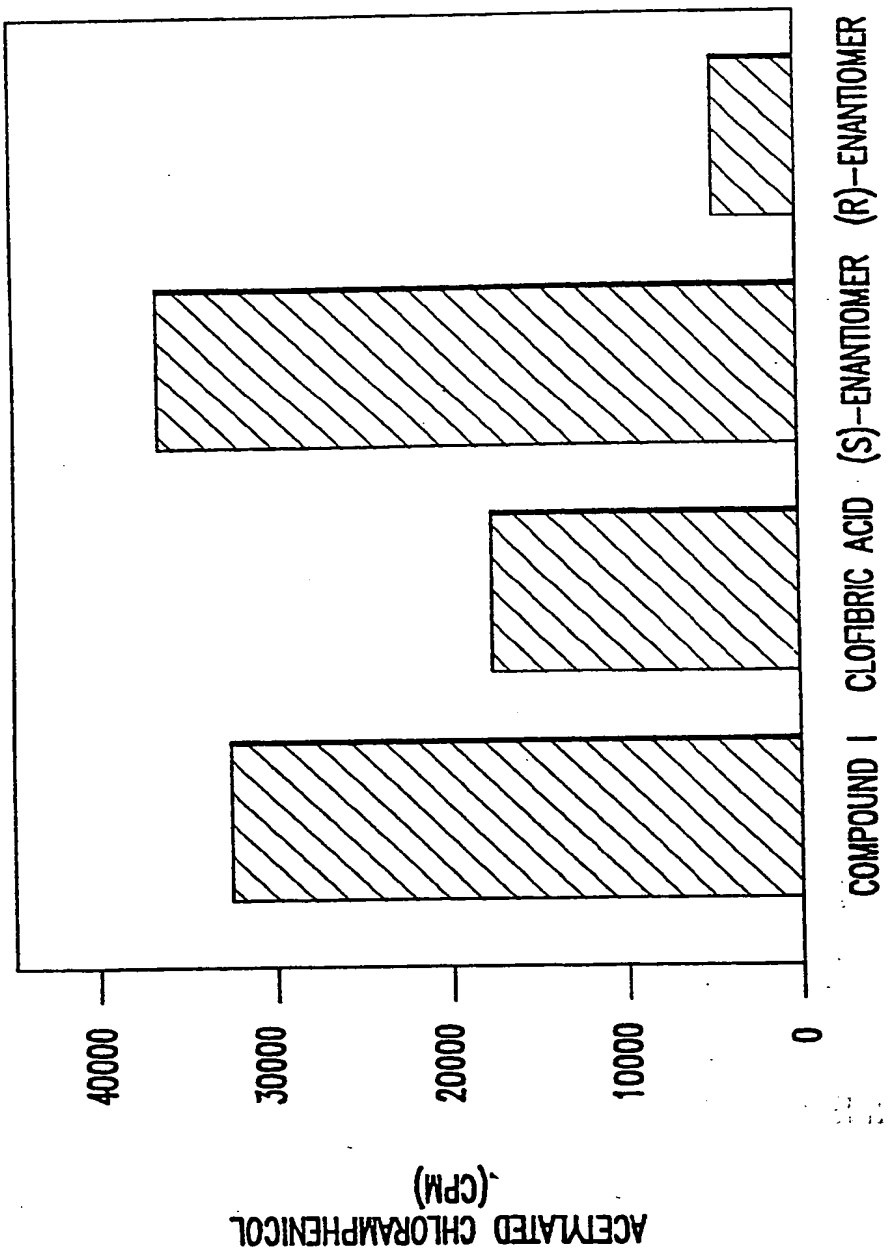
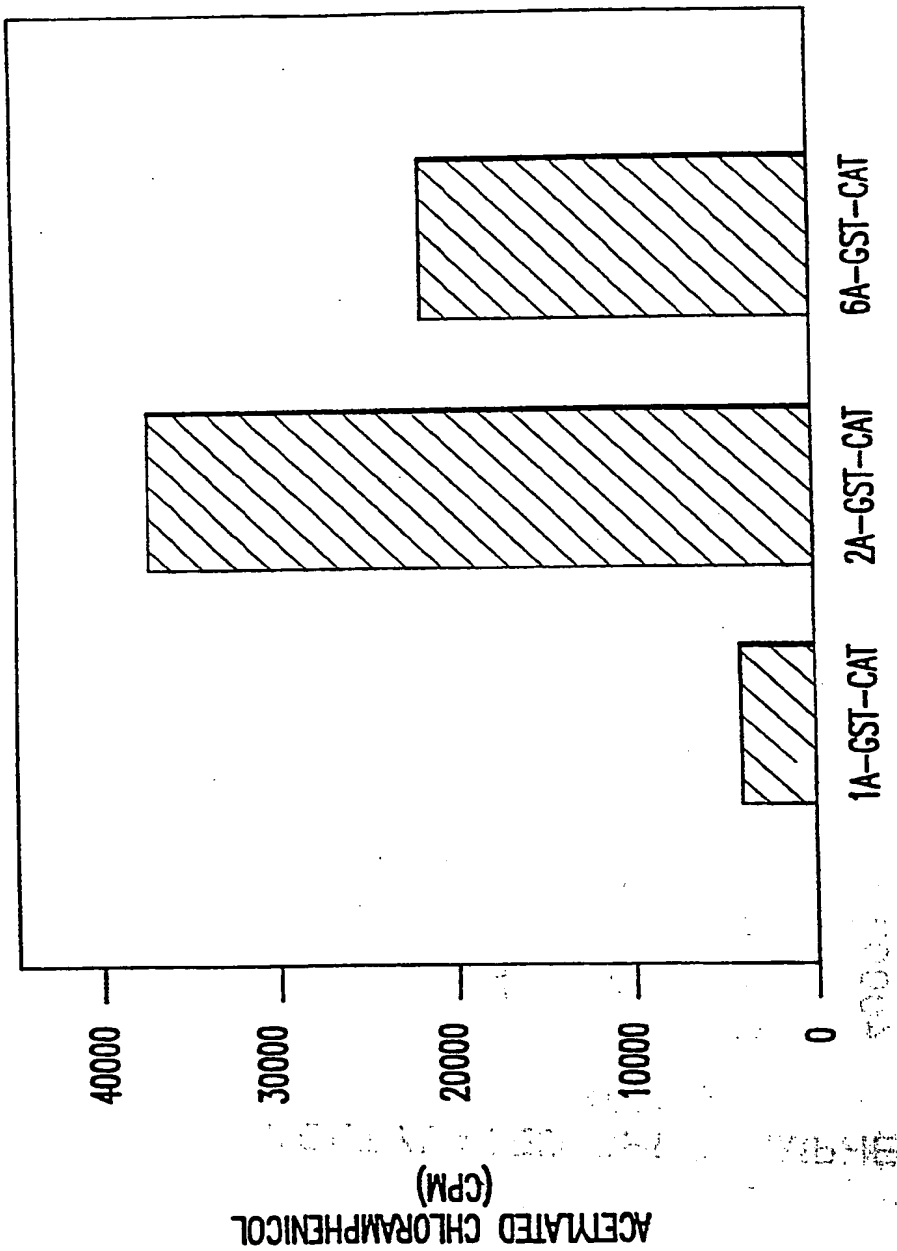


FIG.3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6A-GST-CAT

FIG.4

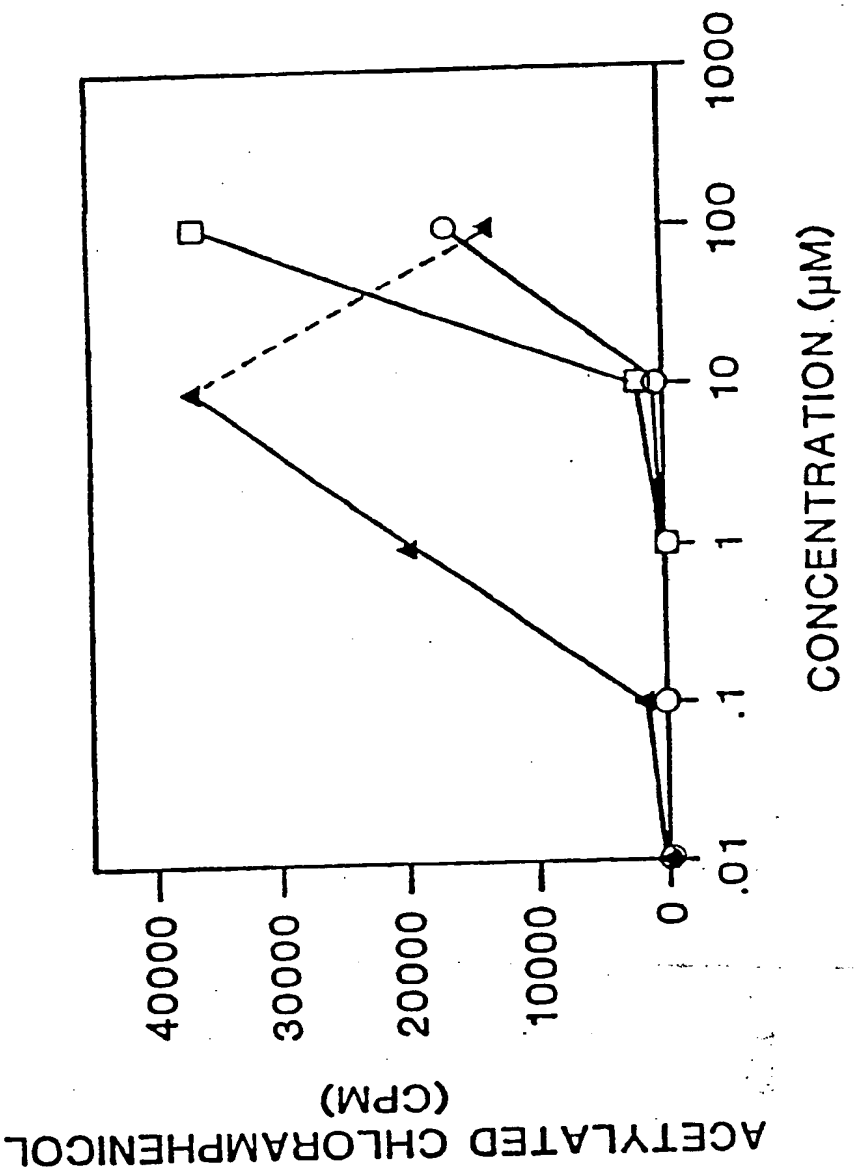


FIG. 5

TITLE OF THE INVENTION

ASSAY TO DETECT PEROXISOME PROLIFERATOR ACTIVITY

BACKGROUND OF THE INVENTION

Peroxisome proliferators are a structurally diverse group of chemicals that, when administered to rodents, not only lead to the induction of peroxisomes but also cause an enlargement of the liver, a decrease in mitochondrial size, a dramatic increase in the peroxisomal capacity for fatty acid oxidation and eventually promote liver tumors. Compounds included in this group are industrial reagents like phthalate esters used as plasticizers and halogenated hydrocarbon solvents as well as a number of hypolipidemic drugs such as clofibrate. The mechanism whereby these nongenotoxic carcinogens cause the development of

hepatocellular carcinomas is still poorly understood. It is proposed that the overexpression of the genes involved in peroxisome biogenesis could play an indirect role by increasing oxidative stress within the cell.

During the course of drug development, many newly synthesized compounds are tested for their ability to cause peroxisome proliferation in the livers of mice and rats as a preliminary screen to preclinical testing. Such an assessment usually requires a significant amount of pure chemical since doses range from 400-800 mg/kg/day, for 4-14 days of treatment using 3-4 animals of both sexes.

While no correlation has been shown between peroxisome proliferation and disease states in higher mammals, peroxisome proliferator activity of a potential pharmaceutical compound is undesirable.

Recently, a new member of the steroid receptor gene super family, peroxisome proliferator activated receptor (PPAR), has been identified which can be activated by known peroxisome proliferators (I. Issemann and S. Green, Nature, 347: 645 (1990)). This was demonstrated using a transactivation assay with a chimeric receptor containing the human estrogen receptor DNA binding domain joined to the ligand binding domain of PPAR (ER-PPAR), and the Vit-G-CAT reporter plasmid. Reporter activity was shown to be ER-PPAR dependent and the level of activity was related to the rank order of potency of the proliferator.

It has also been shown that the promoter region of the rat acyl-CoA oxidase (AOX) gene, the first peroxisomal enzyme in the β-oxidation pathway,

contains cis-regulatory DNA sequences that are peroxisome proliferator responsive (T. Osumi et al., Biochem. Biophys. Res. Communications, 175, 866 (1991)). Two such sequences were identified within the AOX promoter, site A which has a positive regulatory effect and site B which has a negative regulatory effect.

Because there is very little correlation between chemical structure, other than some acidic moiety, and peroxisome proliferation in animals, it would be beneficial to have a rapid assay that would require small quantities of compounds for structure-activity relationship investigations.

DESCRIPTION OF THE FIGURES

Figure 1: Schematic structure of the three reporter constructs tested. One, two and six A elements (□) of the rat acyl-COA oxidase gene (AOX) were fused in the forward orientation (→) upstream of the -164 CAT GST minimal promoter.

Figure 2: Dose-response comparison after co-transfection of pSG5-PPAR and 2A-GST-CAT in COS1 cells and treatment with Wy-14,643 (n=2) (□), nafenopin (n=3) (Δ) and clofibric acid (n=2) (•).

Figure 3: CAT activity in COS1 cells after co-transfection of pSG5-PPAR and 2A-GST-CAT, and treatment with 10 μM of either the racemic mixture of the compound of formula I, clofibric acid, the S-enantiomer of the compound of formula I and the R-enantiomer of the compound of formula I.

Figure 4: Responsiveness of each of the variable length reporter constructs in COS1 cells after co-transfection with pSG5-PPAR and addition of 10 μM nafenopin.

Figure 5: Response to nafenopin of the full length PPAR receptor (Δ), the rat (□) and human (o) glucocorticoid-PPAR (GR-PPAR) chimeric receptors. The MMTV-CAT reporter construct was used with the GR-PPARs.

SUMMARY OF THE INVENTION

The instant invention provides a peroxisome proliferator assay wherein a peroxisome proliferator activated receptor and a reporter plasmid containing a responsive element to the receptor operably linked to a reporter gene, such as chloramphenicol acetyl transferase, are co-transfected into a host cell, the transfected cell is exposed to a chemical entity and reporter activity is measured.

The specificity of the assay of the instant invention for peroxisome proliferators is demonstrated by stereochemical selectivity, using enantiomers of a leukotriene antagonist, where only one of the enantiomers is a peroxisome proliferator in mice.

DEFINITION OF TERMS AND ABBREVIATIONS

Chimeric receptor - a fused protein in which the ligand binding portion from one receptor is combined with the DNA binding portion from another receptor.

Reporter plasmid - a plasmid encoding a reporter gene for an enzymatic activity such that upon expression of the reporter gene and addition of the appropriate substrate a quantifiable product is produced.

CAT - chloramphenicol acetyltransferase

Minimal promoter - the minimum length of DNA to which an RNA polymerase can attach to initiate transcription (the beginning of mRNA synthesis).

DETAILED DESCRIPTION OF THE INVENTION

The instant invention is directed to a cell based transactivation assay useful in detecting peroxisome proliferator activity of a chemical entity. More specifically, the instant invention provides a method for testing a chemical entity for peroxisome proliferator activity which comprises the steps of:

a) providing a host cell which contains a reporter gene operably-linked to a peroxisome proliferator receptor binding responsive promoter and which further contains a peroxisome proliferator activated receptor;

b) contacting the host cell with the chemical entity; and

c) assaying the expression of the reporter gene through detection of a product of expression of the reporter gene.

The term "peroxisome proliferator activated receptor" describes nuclear hormone receptors which are activated in the presence of a peroxisome proliferator. The term includes the full length peroxisome proliferator activated receptor (PPAR), its cDNA, chimeric peroxisome proliferator activated receptors (chimeric PPAR) and the like.

The term "chimeric PPAR" describes a receptor and expression vectors containing regions encoding the putative ligand binding domain of PPAR operably-linked to the DNA-binding domain of an unrelated receptor gene. The PPAR unrelated receptor gene is selected from human estrogen receptor, human glucocorticoid receptor and rat glucocorticoid receptor.

The term "receptor binding responsive promoter" describes a regulatory element that increases the transcription of downstream genes upon binding off the activated receptor. This term includes the "A" site of the peroxisome proliferator responsive region in the promoter of the rat acyl-CoA oxidase gene, the glucocorticoid-responsive region of mouse mammary tumor virus (MMTV), operably-linked multiple copies of these responsive regions, and the like.

The term "reporter gene" represents a gene whose expression can be monitored and whose expression may serve as an indicator of PPAR responsive activity.

In an embodiment of this invention, the reporter gene is a gene not normally expressed by the

host, or is a gene that replaces a gene endogenous to the host.

As used herein the term "host cell" means any cell that is the recipient of a cloning or expression vehicle. The term "host cell" includes a yeast cell, such as Saccharomyces cerevisiae and a cultured animal cell such as a mammalian or insect cell and specifically includes COS1, CV-1, HepG2 and HepA1 cell lines.

As used herein, the term "operably-linked" means that two macromolecular elements are arranged such that modulating the activity of the first element induces an effect on the second element. In this manner, modulation of the activity of a receptor binding responsive promoter may be used to alter and/or regulate the expression of an operably-linked reporter gene. For example, the transcription of a coding sequence of a reporter gene that is operably-linked to a promoter element is induced by factors that "activate" the promoter's activity; transcription of a coding sequence that is operably-linked to a promoter element is inhibitited by factors that "repress" the promoter's activity. Thus, a responsive promoter region is operably-linked to the coding sequence of a reporter gene if transcription of such coding sequence activity is influenced by the activity of the binding site.

An embodiment of the method of the instant invention is a method which comprises the steps of:

a) providing a host cell which contains a reporter gene operably linked to the "A" site of the peroxisome proliferator responsive region in the rat

acyl-CoA oxidase gene promoter; and which further contains the full length peroxisome proliferator activated receptor cDNA;

b) contacting the host cell with a chemical entity having unknown peroxisome proliferator activity; and

c) assaying the expression of the reporter gene through detection of a product of expression of the reporter gene.

A class of this embodiment of the method of the instant invention is the method wherein the host cell is a COS1 cell.

Another class of this embodiment of the method of the instant invention is the method wherein the reporter gene is the -164CAT, which contains the CAT structural gene and glutathione S-transferase Ya subunit minimal promoter under the control of an upstream AOX PPAR responsive element.

For use in the method of the invention, a host cell must possess at least two genetic constructs; a first construct providing genetic sequences capable of expressing a PPAR, which upon the binding of a ligand (in this assay a peroxisome proliferator) induces the expression of a second construct, providing genetic sequences containing a reporter gene. In such a host cell, it is necessary that the promoter of the reporter gene contains the receptor binding element that is recognized by the DNA binding domain of the receptor protein. Thus, expression of the reporter gene is dependent upon expression of the PPAR and presence of a peroxisome proliferator. Once this has occurred, the activated PPAR can bind to the

reporter gene's promoter. The binding of the ligand is therefore quantifiable by measuring the product of the reporter gene, which is also directly correlated with the potency of the peroxisome proliferator.

To express the recombinant fusion constructs of this invention, transcriptional and translational signals recognizable by the host are necessary. A cloned sequence, obtained through the methods well known in the art, may be operably-linked to sequences controlling transcriptional expression in an expression vector, and introduced, for example by transformation or transfection, into a host cell to produce the recombinant receptor proteins or functional derivatives thereof, for use in the methods of this invention.

Transcriptional initiation regulatory signals can be selected that allow for repression or activation of the expression of the fusion construct, so that expression of the fusion construct can be modulated, if desired. Of interest are regulatory signals that are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or that are subject to chemical regulation, for example, by a metabolite or a metabolite analog added to the growth medium.

Alternatively, the fusion construct may be constitutively expressed in the host cell.

Where the native expression control sequences do not function satisfactorily in the host cell, then sequences functional in the host cell may be substituted.

Expression of the fusion constructs in different hosts may result in different post-translational modifications which may alter the

properties of the proteins expressed by these constructs. It is necessary to express the proteins in a host wherein the ability of the protein to retain its biological function is not hindered. Expression of proteins in yeast hosts may be achieved using yeast regulatory signals. The vectors of the invention may contain operably-linked regulatory elements such as enhancer elements (upstream activator sequences in yeast), or DNA elements which confer species, tissue or cell-type specific expression on an operably-linked gene.

In general, expression vectors containing transcriptional regulatory sequences, such as reporter genes and transcription termination sequences, are used in connection with a host. These sequences facilitate the efficent transcription of the gene fragment operably-linked to them. In addition, expression vectors also typically contain discrete DNA elements such as, for example, (a) an origin of replication which allows for autonomous replication of the vector, or, elements which promote insertion of the vector into the host's chromosome in a stable manner, and (b) specific genes which are capable of providing phenotypic selection in transformed cells. Eukaryotic expression vectors may also contain elements which allow it to be maintained in prokaryotic hosts; such vectors are known as shuttle vectors.

The precise nature of the regulatory regions needed for gene expression will vary between species or cell types and there are many appropriate expression vector systems that are commercially available.

Yeast may be used as the host cells. The

elements necessary for transcriptional expression of a gene in yeast have been recently reviewed (Struhl, L. Ann. Rev. Biochem. 58:1051-1077 (1989)). In yeast, most promoters contain three basic DNA elements: (1) an upstream activator sequence (UAS); (2) a TATA element; and, (3) an initiation (I) element. Some promoters also contain operator elements.

Mammalian cells may be used as the host cells. A wide variety of transcriptional and translational regulatory signals can be derived for expression of proteins in mammalian cells and especially from the genomic sequences of viruses which infect eukaryotic cells.

Once the vector or DNA sequence containing the construct(s) is prepared for expression, the DNA construct(s) is introduced into an appropriate host cell by any of a variety of suitable means, for example by transformation or transfection. After the introduction of the vector, recipient cells are treated with the chemical entity to be assayed.

If the chemical entity is a peroxisome proliferator which binds to the receptor, the reporter gene will consequently be expressed. The extent of this expression may be measured in many ways. The product of the reporter gene may be directly assayed with an immunoassay. Such immunoassays include those wherein the antibody is in a liquid phase or bound to a solid phase carrier. In addition, the reporter gene product can be detectably labeled in various ways for use in immunoassays. Immunoassays for detecting a reporter protein include radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), or other assays known in

the art, such as immunofluorescent assays, chemiluminescent assays, or bioluminescent assays.

Any detectable phenotypic change may serve as the basis for the detection methods of this invention. Especially useful are reporter genes that confer a new phenotype on the host when expressed. Genes that endow the host with an ability to grow on a selective medium are especially useful. For example, in yeast, use of the yeast LEU2 gene as a reporter gene in mutant strains that lack endogenous LEU2 activity allows such yeast to grow on leucine as a sole carbon source (see, e.g., The Molecular Biology of the Yeast Saccharomyces, ed. Strathern et al., Cold Spring Harbor Press, Cold Spring Harbor, NY 1981; and the methods described in Rothstein et al., Mol. Cell Biol. 7:1198, 1987). Expression of the reporter gene is monitored by merely observing whether the host retains the ability to grow on leucine.

The E. coli lacZ gene is also very useful as a reporter. In hosts which utilize the lacZ gene as a reporter gene, expression and activity of the fusion protein can be easily scored by monitoring the production of β-galactosidase. The production of β-galactosidase may be visually monitored in the presence of a chromophoric dye, such as X-gal, which turns blue after hydrolysis by the enzyme.

Other reporter genes include his3, ura3, trp1 and, in mammalian cells, luciferase, alkaline phosphotase and chloramphenicol acetyltransferase (CAT). CAT activity is easily measurable, and directly correlates with the level of CAT gene expression.

While the present invention comprehends a transient cell-based assay, a cell line that stably expresses the genes utilized in a peroxisome proliferation assay is also intended. Expression of the cloned gene sequence(s) results in the production of the PPAR or in the production of a fragment of this peptide. This expression can take place in a continuous manner in the transformed cells, or may be induced by exogenous agents.

Preferably, genetically stable transformants are constructed by integrating the PPAR into the chromosome of the host. Such integration may occur, through homologous recombination, following transformation with the exogenous DNA, or it may depend upon a vector that functionally inserts itself into the host chromosome, for example, using retroviral vectors, transposons or other DNA elements that promote integration of DNA sequences into chromosomes. Alternatively, the PPAR gene may be introduced on a vector which does not replicate; expression of the fusion protein continues so long as the vector persists in the cell population. Thus after the introduction of the vector or DNA sequence into the cell, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells.

Cells that have been transformed with the fusion protein DNA vectors of the invention may be selected, under constant positive selection, by also introducing one or more markers that allow for selection of host cells that contain the vector. For example, the marker may provide an amino acid synthesis

enzyme or biocide resistance, e.g. resistance to antibiotics, such as G418, or heavy metals, such as copper, or the like.

A cell line that stably expresses the genes utilized in the instant assay may also be recognized by performing the assay with the cell line using a known potent peroxisome proliferator (such as Wy-14,643) as a substrate.

The method of this invention provides a rapid, reliable and economic manner in which to screen and classify compounds as peroxisome proliferators. The method of the invention is amenable to large scale industrial screening of large numbers of compounds or preparations for peroxisome proliferator activity. In addition, the method of the invention allows the screening of any pure compound, mixture of compounds, or mixture of preparations, so as to identify the additive, synergistic, or detrimental effects of such compositions.

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations, modifications, deletions or additions of procedures and protocols described herein, as come within the scope of the following claims and its equivalents.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1

Peroxisome Proliferator activated receptor (PPAR)

The peroxisome proliferator activated receptor (PPAR) cDNA was cloned by polymerase chain reaction (PCR) amplification (as described by R. K. Saiki et al., Science, 239, 487 (1988)) from a mouse liver cDNA library (Clonetech). The 5' primer used was "CCACCATGGTGGACACAGAGAGCCCCATC" (sequence ID :1:) and contained a consensus Kozak sequence, CCACC, just before the ATG initiation condon. The 3' primer was "GGATCGATCAGTACATGTCTCTGTAGATCTC" (sequence ID :2:). Amplification was carried out using 3 µl of the library as template and 50 picomoles of primers in a total volume of 50 µl. The conditions for amplification were 94°C for 30s, 55°C for 45s and 72°C for 2 min., for 35 cycles. The appropriate PCR products were then cloned into pBluescript SK+ and three of the full-length PPAR cDNA clones were sequenced. All were found to have at least three random sequence errors introduced by the TAQ polymerase during amplification. However, all clones contained a common change in nucleotides 391 and 392, CG changed to GC, which resulted in the substitution of arginine for alanine at amino acid residue 75. The full-length PPAR cDNA employed herein was identical to the published sequence (Nature, 347, 654 (1990)) except for the CG change at position 391-2. The full-length PPAR cDNA was cloned into the eukaryotic expression vector pSG5 (Stratagene) (pSG5-PPAR) for transfection experiments.

EXAMPLE 2

The human glucocorticoid receptor-PPAR chimeric receptor was constructed essentially as described by Issemann and Green (Nature, 347, 645 (1990)). Briefly, the fragment containing the DNA binding domain codons 1-486 of the human glucocorticoid receptor was fused to the mouse PPAR ligand binding domain containing codons 167-469. The hGR DNA binding domain was obtained by reverse transcription-PCR using 1 μg of HepG2 poly A RNA as the template and the following oligonucleotide primers: 5' primer CCACCATGGACTCCAAAGAATCA (sequence ID :3:) and 3' primer TTCTCGAGCCATTCCAGCCTGAAGACA (sequence ID :4:). A Xho 1 restriction sequence was included in the 3' primer downstream of codon 486 of the hGR in order to facilitate ligation to the PPAR ligand binding domain (see below). The 1.4kb PCR product containing the hGR DNA binding domain was cloned into the EcoRV site of pBluescript SK(+) and sequenced (pSK(+)/GR). The PPAR ligand binding domain was obtained by PCR using a mouse liver cDNA library and the following oligonucleotide primers: 5' primer CGCTCGAGAATCACACAATGCAATTCGCTTTGGA (sequence ID :5:) and 3' primer GGATCGATCAGTACATGTCTCTGTAGATCTC (sequence ID :6:). A Xho 1 restriction sequence was included in the 5' primer upstream of codon 167 of the PPAR. The 0.9 kb PCR product containing the mouse PPAR ligand binding domain was cloned into the EcoRV site of pBluescript SK(+) and sequenced (pSK(+)/PPAR). The plasmid pSK(+)/GR-PPAR fusion was constructed by ligating the Xho 1 fragment isolated from pSK(+)/GR into the Xho 1

site of pSK(+)/PPAR. To obtain the hGR-PPAR chimeric receptor expression vector the Asp 7181-BamH1 fragment from pSK(+)/GR-PPAR was isolated, Klenow blunt ended and inserted into pSG5 expression vector at the BamH1 site which was also blunt ended with Klenow.

The rat GR-PPAR chimeric receptor was prepared in an identical fashion except that the fragment containing the DNA binding domain of the rat glucocorticoid receptor was obtained by 20 cycles of PCR using 10 ng of pRSV-ratGR (described by R. Miesfield et al., Cell, 46, 389 (1986)) as the template and the following primers: 5' primer CCACCATGGACTCCAAAGAATCCTTAGCT (sequence ID :7:) and 3' primer TTCTCGA GCCATTCCAGCCTGAAGACATTTCCG (sequence ID :8:). Also the Xho 1 restriction sequence was included in the 3' primer downstream of codon 505 of the rat glucocorticoid receptor.

EXAMPLE 3

Acyl-CoA oxidase - CAT reporter plasmid

Part A: Peroxisome proliferator responsive region in acyl-CoA oxidase gene

Oligonucleotides for the top and bottom strands of the "A" site (top strand-TTCCCGAACGTGACCTTTGTCCTGGTCCCCT (sequence ID :9:)) of the peroxisome proliferator responsive region in the acyl-CoA oxidase gene (T. Osumi et al., Biochemical and Biophysical Research Comm., 175, 866 (1991)) were synthesized on an Applied Biosystems 380B synthesizer. The strands were gel purified as described by T. H.

Rushmore et al., J. Bio Chem., 266, 11632 (1991). Specifically the oligonucleotides were purified by electrophoresis on a 12% denaturing polyacrylamide-urea gel (acrylamide: bisacrylamide, 30:1) in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). The bands in the gel were identified by u.v. shadowing, eluted from the gel overnight in 5 mL of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA), desalted, and concentrated by passage through an OPC purification column (Applied Biosystems). The purified oligonucleotides were stored as single strands in water at a concentration of 1 µg/µL at -20°C.

Part B: -164 CAT GST Ya minimal promoter

The minimal promoter - -164 CAT GST Ya was prepared as described by Rushmore et al. (Proc. Natl. Acad. Sci U.S.A., 87, 3826 (1990) and has the following sequence (seq. I.D. :10:):

-164

5' - cttgtcagcccctcccccagtttgtcacctttcgagtctatcacgaaag
tctggaatcttggactctatgggtgtctgtgggaagggctgttccctattgg -63
tccccacaccctgggtaagaattgtcaccatattaaagtggcgtgcacactc -11
ctctggagctGGAGTTGGGAGCTGAGTGGAGAAGAAGCCACGACTCTCGCTAGgtc
+1
agtactctctttacaaccc - 3'.

The -164 CAT GST Ya promoter may also be prepared by oligonucleotide synthesis well known in the art, in which complementary oligonucleotides are hybridized and double stranded DNA provided by Klenow fill in.

Part 3: Acyl - CoA oxidase - CAT reporterplasmid

The oligonucleotides described in Part A were phosphorylated, annealed and ligated into the blunt-ended Nde I site of the -164 CAT GST Ya promoter described in Part B.

EXAMPLE 4

Employing the procedure described in Example 3 but varying the number of peroxisome proliferator response regions of the acyl-CoA oxidase gene operably attached to the -164CAT reporter provided response/reporter plasmids having the general structures illustrated in Figure 1.

EXAMPLE 5

General Procedure for Cell Transfection

Monkey kidney cell line COS1 cells (ATCC) were maintained in phenol red free Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 5% fetal calf serum stripped of endogenous steriods (CBI). The cells were plated in 10 cm dishes at a density of 5×10^5 cells/dish and transfected after 24 hours. The cells were transfected according to the procedure described by C.A. Chen and H. Okayama, Biotechniques, 6, 632 (1988). Specifically, the exponentially growing cells are trypsinized and seeded at 5×10^5 cells/10 cm plate/10 mL of growth medium and incubated overnight. Plasmid DNA in 1-30 µL is mixed with 0.5 mL of 0.25 M $CaCl_2$ and 0.5 mL of 2 x BBS (50 mM N,N-Bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid,

280 mM NaCl, 1.5 mM Na_2HPO_4, pH adjusted to 6.95) is added. The mixture is incubated at room temperature for 10-20 mins. The calcium phosphate-DNA mixture is then added to the cell plate dropwise and the plate swirled well. The plate is incubated overnight at 35°C in a 3% CO_2 atmosphere.

EXAMPLE 6

General Procedure for Chemical Entity Assay

The transfected cells obtained as described in Example 5 were rinsed twice with phosphate buffered saline (PBS) and then a medium containing the chemical entity to be assayed was added. The plates were incubated for 48 hours. After the first 24 hours of incubation fresh medium containing the test compound was added. At the end of the 48 hours the cell medium was removed by aspiration and the attached cells were washed with PBS (Mg^{2+} and Ca^{2+} -free). The cells were then scraped from the dish, recovered by centrifugation and resuspended in 0.25 Tris, pH 8.0. The cells were then lysed by three to four freeze-thaw cycles, heated for 10 minutes at 60°C and the lysates cleared by centrifugation. 5-10µL of the cleared lysate was incubated with C^{14}-chloramphenicol and N-butyryl Coenzyme A for 30-60 minutes. The butylated chloramphenicol was recovered by organic extraction and the extract was analyzed by liquid scintillation. The results were expressed as counts per minute (CPM) acylated chloramphenicol/µg of protein at various

concentrations of the assay compound minus background activity (DMSO).

Using the general procedure described above the following compounds were tested for peroxisome proliferator activity using cells transfected with full length PPAR and acyl-CoA oxidase - CAT reporter plasmid. The activity of the compounds is given in concentration of ED_{50} based on the maximum response concentration.

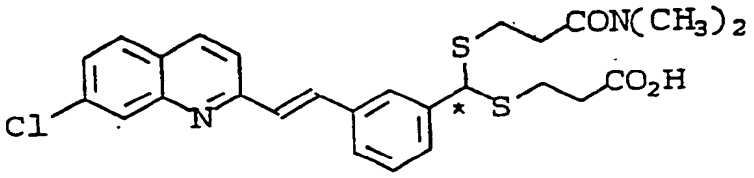
	Activity
Wy-14,643	0.04μM
nafenopin	0.8μM
clofibric acid	6μM

These values were derived from the dose-response data illustrated in Figure 2.

EXAMPLE 7

Stereoselective Sensitivity of Assay

Using the general assay procedure described in Example 5, the racemic mixture of the compound having the formula I as shown below as well as its two seperate enantiomers were evaluated. The comparative results are shown in Figure 3. The graph shows that virtually all of the peroxisome proliferator activity of the racemic mixture resides in only one of the enantiomers (S-enantiomer). This result is the same as that found when these compounds were tested using the mouse in vivo assay.



I

(* = prochiral center)

EXAMPLE 8

The transfection and assay procedures described in Examples 5 and 6 were employed but the 2A-GST-164 CAT was replaced by plasmids having one response region and 6 response regions of the acyl-CoA oxidase. Comparative results of the assays of 10 μM nafenopin in cells containing the various plasmids are shown in Figure 4. The graph shows that the maximum sensitivity for the assay of the instant invention occurs when the promoter incorporates 2 units of the acyl-CoA oxidase responsive region.

EXAMPLE 9

The transfection and assay procedures described in Examples 5 and 6 were employed but the full length PPAR gene was replaced with rat glucocorticoid-PPAR chimeric receptor or human glucocorticoid-PPAR chimeric receptor prepared as described in Example 2. Comparative results of the assays of 10μM nafenopin in cells comprising the

various receptor combinations are illustrated in Figure 5. The results show that assay employing the full length PPAR has significantly greater sensitivity than the assays employing the chimeric PPAR receptors.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCACCATGGT GGACACAGAG AGCCCCATC 29

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGATCGATCA GTACATGTCT CTGTAGATCT C 31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCACCATGGA CTCCAAAGAA
TCA 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTCTCGAGCC ATTCCAGCCT
GAAGACA 27

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCTCGAGAA TCACACAATG CAATTCGCTT TGGA 34

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGATCGATCA GTACATGTCT CTGTAGATCT C 31

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCACCATGGA CTCCAAAGAA TCCTTAGCT 29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTCTCGAGCC ATTCCAGCCT GAAGACATTT CCG 33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTCCCGAACG TGACCTTTGT CCTGGTCCCC
T 31

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 230 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTTGTCAGCC CCTCCCCCCA GTTTGTCACC TTTCGAGTCT ATCACGAAAG
TCTGGAATCT 60

TGGACTCTAT GGGTGTCTGT GGGAAGGGCT GTTCCCTATT GGTCCCCACA
CCCTGGGTAA 120

GAATTGTCAC CATATTAAAG TGGCGTGCAC ACTCCTCTGG AGCTGGAGTT
GGGAGCTGAG 180

TGGAGAAGAA GCCACGACTC TCGCTAGGTC AGTACTCTCT
TTTACAACCC 230

WHAT IS CLAIMED IS:

1. A method for testing a chemical entity for peroxisome proliferator activity which comprises the steps of:

a) providing a host cell which contains a reporter gene operably-linked to a peroxisome proliferator receptor binding responsive promoter and which further contains a peroxisome proliferator activated receptor;

b) contacting the host cell with the chemical entity; and

c) assaying the expression of the reporter gene through detection of a product of expression of the reporter gene.

2. The method according to Claim 1 wherein the peroxisome proliferator activated receptor is full length peroxisome proliferator activated receptor.

3. The method according to Claim 1 which comprises the steps of:

a) providing a host cell which contains a reporter gene operably linked to at least one of the "A" site of the peroxisome proliferator responsive region in the rat acyl-CoA oxidase gene; and which further contains the full length peroxisome proliferator activated receptor cDNA;

b) contacting the host cell with the chemical entity; and

c) assaying the expression of the reporter gene through detection of a product of expression of the reporter gene.

4. The method according to Claim 3 wherein two "A" sites of the peroxisome proliferator responsive region in the rat acyl-CoA oxidase gene are operably linked to the promoter gene.

5. The method according to Claim 3 wherein the reporter gene is a GST Ya minimal promoter linked to CAT.

6. A cell useful for testing a chemical entity for peroxisome proliferator activity which contains a reporter gene operably-linked to a receptor binding responsive promoter and which further contains a peroxisome proliferator activated receptor.

7. The cell according to Claim 6 wherein the peroxisome proliferator activated receptor is full length peroxisome proliferator activated receptor.

8. The cell according to Claim 6 which contains a reporter gene operably linked to the "A" site of the peroxisome proliferator responsive region in the rat acyl-CoA oxidase gene; and which further contains the full length peroxisome proliferator activated receptor cDNA.

9. The cell according to Claim 6 wherein two "A" sites of the peroxisome proliferator responsive region in the rat acyl-CoA oxidase gene are operably linked to the promoter gene.

10. The cell according to Claim 6 wherein the reporter gene is a GST Ya minimal promoter linked to CAT.

Patents Act 1977
Examiner's report to the Comptroller under Section 17
(The Search report)

Application number
GB 9316410.1

Relevant Technical Fields

(i) UK Cl (Ed.)

(ii) Int Cl (Ed.)

Search Examiner
DR D ELSY

Date of completion of Search
8 NOVEMBER 1993

Databases (see below)

Documents considered relevant following a search in respect of Claims :-
1-10

(ii) ONLINE DATABASES: WPI, DIALOG (BIOTECH)

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages	Relevant to claim(s)
X,Y	Biosis Abstract Number: 94107534 and Proc Natl Acad Sci (USA), Vol 89, No 16 (1992), pages 7541-7545 ZHANG B ET AL	X: 1, 2, 6, 7 Y: 3, 4, 8, 9
Y	Biosis Abstract Number: 93122532 and EMBO J, Vol 11, No 2 (1992), pages 433-440 TUGWOOD J D ET AL	3, 4, 8, 9

Databases: The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).